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Four New Genotypes of Adenovirus Type 3 Isolated From Patients With Conjunctivitis in Japan

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Adenovirus type 3 (Ad3) was the most frequently isolated serotype from patients with conjunctivitis during the period from 1989 to 1991 in Japan. All Ad3 strains isolated in 1990 had an identical genotype, Ad3f. However, in 1992, the predominant serotype was replaced by Ad4. The genome type was examined to determine whether genetic changes existed in Ad3 isolates in 1992. Ad3 isolated from 55 patients with acute conjunctivitis during the period from June 1992 to February 1993 in Japan was assessed by genome typing with restriction endonucleases *Bam*H I, *Bgl* II, *Hin*d III, and *Sma* I recognizing 6-base-pair sequences. The emergence of four new genotypes of Ad3 was identified; one with a new *Bam* HI site (one isolate), one with a new *Bgl* II site (two isolates), one with a new *Hin*d III site (three isolates) and one with a new *Sma* I site (two isolates). This study demonstrates that the emergence of a new genotype of Ad3 may contribute to the replacement of the predominant serotype associated with adenovirus conjunctivitis in Japan. ***J. Med. Virol. 59:73–77, 1999.***

KEY WORDS: adenovirus; Ad3; conjunctivitis; genome-RFLP; Japan; epidemiology

INTRODUCTION

Human adenoviruses cause various diseases, such as acute respiratory, ocular, gastrointestinal and urinary tract infections. To date, 49 serotypes have been identified [Hierholzer et al., 1988; Schnurr and Dondero, 1993]. Adenovirus types 3, 4, 7, 8, 19, and 37 have been found to be responsible for the majority of adenovirus conjunctivitis cases in Japan [Yamadera et al., 1995]. Adenovirus is known to be one of the most common causes of pharyngoconjunctival fever (PCF) in children and epidemic keratoconjunctivitis (EKC) in adults.

The Ad3 genotype recovered from most conjunctivitis cases in Japan in 1983 was Ad3g [Guo et al., 1988]; however, this changed to Ad3f in 1986 [Itakura et al., 1990]. In 1990, all 45 isolates of Ad3 in Japan from patients with acute conjunctivitis were Ad3f [Shiao et al., 1996]. No Ad3g was detected in the same year. Adenovirus type 3 was not only the predominant serotype recovered from ocular infections during the period between 1989 and 1991, but also the most frequently isolated serotype among the adenoviruses that cause various diseases such as acute respiratory, gastrointestinal, and urinary tract infections in Japan from 1986 to 1992 [NIH, 1991, 1994, 1995; Yamadera et al., 1995].

Recently, many adenovirus strains have been classified by restriction endonuclease (RE) analysis. RE analysis has also been used to establish the relationship between strains of Ad3 in a variety of diseases on different continents [Adrian et al., 1986; Li and Wadell, 1988].

The emergence of new Ad3 genotypes associated with acute conjunctivitis in Japan in 1992 is described.

MATERIALS AND METHODS

Patients

Strains of Ad3 were isolated from the conjunctival swabs of 55 patients who attended 13 eye clinics in eight prefectures of Japan from June 1992 to February 1993. Clinical records were available for 33 of the 55 patients studied. Eighteen of the 33 patients (54.5%) had a diagnosis of EKC, and 15 (45.5%) had a diagnosis of PCF. The geographic distribution of the patients is listed in Table I.

Inoculation and Identification of Adenoviruses

All strains were propagated in HEp-2 cells and identified as Ad3 by neutralization test with rabbit hyper-

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TABLE I. Geographic Distribution of Patients With Adenovirus Type 3 Conjunctivitis in 1992

Prefecture	No. of strains of genotype					
	Ad3f	Ad3h	Ad3i	Ad3j	Ad3k	Total
Iwate	2					2
Tokyo	21			3		24
Kanagawa	3				1	4
Osaka	1					1
Ehime	2				1	3
Fukuoka	5					5
Kumamoto	10	1	2			13
Okinawa	3					3

immune serum. A cell monolayer was cultivated in a 25-cm^2 culture flask (Costar, New York) containing 5 ml Eagle's modified essential medium (MEM; Nissui, Tokyo, Japan) with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 100 U/ml nystatin). Inoculated cells were incubated for 1 hr with rocking every 15 min. The medium was then replaced with 4% fetal calf serum Eagle's MEM containing the same antibiotics and the flask was incubated for 48–72 hr in an incubator at 37°C with 5% CO_2.

DNA Extraction and Endonuclease Digestion

Viral DNA was prepared from the inoculated cells with phenol and precipitated with isopropanol as described previously [Itakura et al., 1990]. The strain GB of Ad3 obtained from American Type Culture Collection (ATCC, Rockville, MD) was used as the prototype (genotype Ad3p). Four restriction endonucleases recognizing 6-base-pair sequences, *Bam*H I, *Bgl* II, *Hin*d III, and *Sma* I, were used to cut the DNA from the adenovirus isolates according to the protocol recommended by the manufacturer (Takara Shuzo Co., Kyoto, Japan). The DNA fragments were separated by electrophoresis in 1.2% horizontal agarose gels, which were prepared and run in 10 mM Tris-acetate buffer (pH 8.0) with 1 mM ethylenediamine tetraacetic acid (EDTA) at 100 V. After staining with ethidium bromide (0.5 μg/ml), DNA bands in the gels were photographed with an ultraviolet transilluminator.

RESULTS

Genotyping of Ad3 Isolates

Representative patterns of DNA from Ad3 isolates cut with *Bam*HI, *Bgl* II, *Hin*d III, and *Sma* I are shown in Figure 1. Identical restriction patterns were obtained with 47 isolates and these were identified as Ad3f [Guo et al., 1988]. Eight isolates produced unique restriction profiles. A new genotype identified with *Bam*H I was found in one isolate, with *Bgl* II in two, with *Hin*d III in three, and with *Sma* I in two. These isolates were tentatively designated Ad3h, Ad3i, Ad3j, and Ad3k, respectively.

A schematic presentation of the DNA restriction patterns of representative new genotypes is shown in Figure 2. The proposed genotype Ad3h appeared to lack a *Bam*H I restriction site between fragments A and one of the D fragments in Ad3f. The second variant of genotype Ad3i appeared to have lost a *Bgl* II site between fragments C and I of the Ad3f and prototype viruses. The third variant (Ad3j) appeared to have a smaller fragment B than Ad3f and Ad3p. The fourth variant (Ad3k) appeared to lack the *Sma* I restriction site between double fragments of C in Ad3f. Each new genotype was identified as having a change in one restriction enzyme and the digestion patterns from the other three enzymes were the same as Ad3f.

Distribution of Genotypes of Ad3

The total numbers of isolates and new genotypes are shown in Table I, together with their geographical location. The chronological distribution of adenovirus type 3 genotypes compared with previous reports is shown in Table II. One Ad3h and two Ad3i were detected in the same clinic in Kumamoto prefecture located at the southern part of Japan. All three Ad3j were detected in the same clinic in metropolitan Tokyo. However, the two Ad3k were detected in different clinics. One was isolated in a clinic in Kanagawa prefecture and the other in a clinic in Ehime prefecture. Ad3 was the most prevalent serotype isolated in metropolitan Tokyo and in Kumamoto prefecture during the study period (data not shown). Ad3 was isolated from 24 of 39 patients (62%) with adenoviral conjunctivitis in Tokyo and 13 of 30 isolates (43%) in Kumamoto prefecture. In contrast to the high prevalence of Ad3 in Tokyo and Kumamoto, the other two prefectures in which new genotypes were detected showed a lower prevalence than that in Tokyo and Kumamoto. Four of 31 adenovirus isolates (13%) in Kanagawa and 3 of 18 isolates (17%) in Ehime were Ad3.

DISCUSSION

The emergence of four new Ad3 genotypes associated with acute conjunctivitis in 1992 in Japan was demonstrated. During the period from 1983 through 1990, the most prevalent Ad3 genotype associated with conjunctivitis in Japan changed gradually from Ad3g to Ad3f [Guo et al., 1988; Itakura et al., 1990]. However, four new variants were observed in 1992. In 1990, a nationwide epidemiological surveillance of infectious conjunctivitis in Japan detected only one genotype, Ad3f, in 45 isolates of Ad3 [Shiao et al., 1996]. In contrast to the finding of only one genotype of Ad3, Ad3f, in 1990, it is notable that we have identified the emergence of four new genotypes in 8 of 55 Ad3 isolates in this study, from patients with acute conjunctivitis only 2 years after the former investigation. The results of the present study suggest that a significant environmental or host factor may have promoted this change. Alternatively, these new Ad3 genotypes may have been introduced from outside Japan.

Changing appearance of different genotypes of Ad3 has occurred in other countries. In Glasgow, Scotland, O'Donnell et al. [1993] reported that Ad3p was isolated more frequently than other Ad3 genotypes from patients with conjunctivitis during the period from 1981 to 1988, except in 1986 when Ad3a was the most preva-

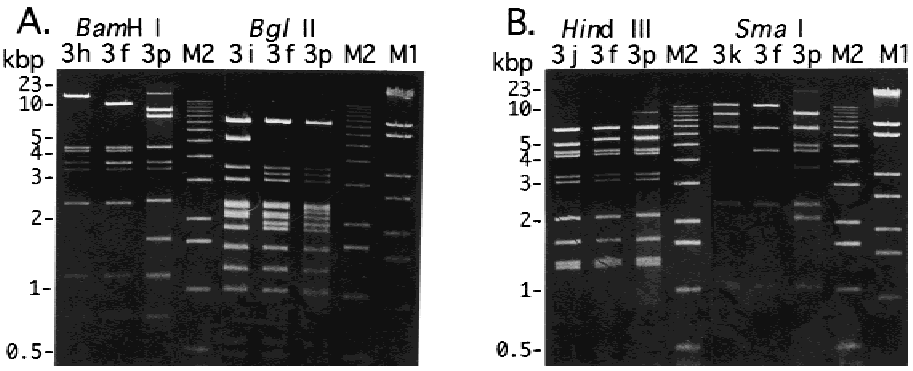

Fig. 1. Restriction enzyme digests of DNA from four new variants, Ad3f and Ad3 prototype. Virus DNA was digested with **(A)** *Bam*H I and *Bgl* II or **(B)** *Hin*d III and *Sma* I. Gel electrophoresis was performed in 1.2% agarose gel. Lane M1, molecular weight marker lambda *Hin*d III digestion; lane M2, molecular weight marker 1 kbp ladder; 3h, Ad3h; 3i, Ad3i; 3j, Ad3j; 3k, Ad3k; 3f, Ad3f; 3p, Ad3 prototype. Restriction enzymes used are indicated above the column. Molecular weight calculated from M1 and M2 is indicated to the left of the column.

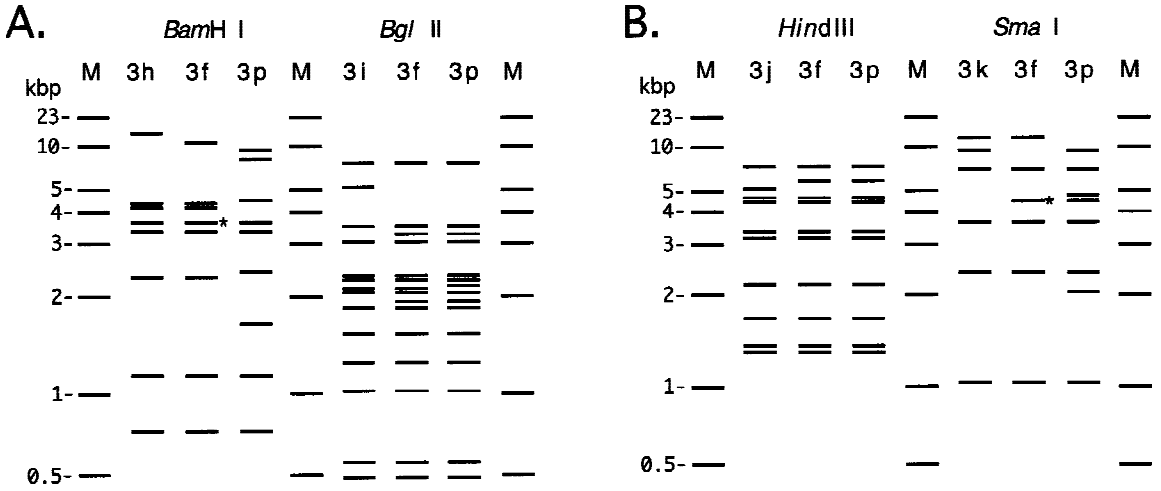

Fig. 2. Schematic presentation of DNA restriction patterns of Ad3 prototype, Ad3f, and isolated new genotypes of Ad3 strains digested with **(A)** *Bam*H I and *Bgl* II or **(B)** *Hin*d III and *Sma* I. Lane M, molecular weight marker obtained from the combination of lambda *Hin*d III and 1 kbp ladder; 3h, Ad3h; 3i, Ad3i; 3j, Ad3j; 3k, Ad3k; 3f, Ad3f; 3p, Ad3 prototype. Restriction enzymes used are indicated above the column. Molecular weight calculated from M is indicated to the left of the column. Asterisk indicates double fragments.

lent genotype. Ad3a was the most frequently isolated genotype between 1989 and 1991. They reported that the appearance of Ad3a occurred simultaneously with an increased number of Ad3 isolates. In Japan, there was an apparent shift from Ad3g to Ad3f as the predominant genotype during the period from 1983 to 1990. In 1990, Ad3 was the most frequently isolated serotype (35%) from patients with conjunctivitis in Japan [NIH, 1995]. However, in 1992, together with the emergence of a new genotype of Ad3, the predominant serotype was replaced by Ad4 [Nakagawa et al., 1995]. In the case of Ad4, it is also suggested that the increased frequency of isolation of serotype Ad4 was due to the emergence of a new genotype, Ad4a, which was more likely to cause conjunctivitis than the prototype strain [Tullo and Higgins, 1980; Aoki et al., 1982].

Two cases of Ad3k identified as a new genotype with *Sma* I were detected in different clinics. One isolate was detected in a clinic in Ehime prefecture on 17th September and the other in Kanagawa prefecture 500 miles away, on 17th December. It is unlikely that the same genomic mutation had occurred in these two strains isolated in different regions of the country within only 3 months. It seems likely, therefore, that the Kanagawa strain was transported from Ehime.

Ad3 is isolated not only in conjunctivitis but also in acute respiratory infection in children. Mizuta et al. [1994] isolated Ad3 from children with acute respiratory infection from 1986 to 1991 in Yamagata, Japan, and found that IIC1a was predominant during the study period. Li et al. [1996] found that Ad3a2 was predominant in children with pneumonia over a 27-year period (1962–1988) in China, except in 1984 when Ad3a4 was the most prevalent genotype. In contrast, Guo et al. [1988] and Shiao et al. [1996] identified solely Ad3f in patients with conjunctivitis in 1986 and 1990, respectively. Itakura et al. [1990] also reported that Ad3f predominated (84%) in conjunctivitis in 1986

TABLE II. Chronological Distribution of Adenovirus Type 3 Genotypes in Japan

Year	No. of strains of genotype							Reference
	Ad3f	Ad3g	Ad3h	Ad3i	Ad3j	Ad3k	Total	
1983	3	6					9	Guo et al.
	4	16					20	Itakura et al.
1986	10						10	Guo et al.
	16	3					19	Itakura et al.
1990	45						45	Shiao et al.
1992	47		1	2	3	2	55	

in Japan. Furthermore, we observed that Ad3f was still the predominant genotype (85%) in 1992 in Japan. Despite the different terminology used in each laboratory, genome digestion patterns of Ad3f were different from those of IIC1a reported by Mizuta et al. and Ad3a2 and Ad3a4 described by Li et al. using four REs, *Bam*H I, *Bgl* II, *Hin*d III, and *Sma* I. These results suggest that the predominant genotypes of Ad3 in respiratory infection and conjunctivitis in Japan are different. Whereas the serotype most frequently causing conjunctivitis in Beijing is unknown, it is suggested that Ad3f and its derivative genotypes are likely to be associated with the conjunctiva rather than with the pharyngeal region.

In the present study, the aim was the detection of the chronological changing of genotype of Ad3 in Japan. Restriction endonuclease analysis using four enzymes was sufficient to compare the isolates with the strains reported previously, because in Japan, these four enzymes (*Bam*H I, *Bgl* II, *Hin*d III, and *Sma* I) have been used to detect changing genotypes of Ad3 [Guo et al., 1988; Itakura et al., 1990; Mizuta et al., 1994; Shiao et al., 1996]. Each new genotype was identified as a change in one restriction enzyme and their digestion patterns with the other three enzymes were the same as that for Ad3f. The changed digestion pattern in each enzyme was distinct from the pattern of all the other Ad3 isolates presented in the literature [Li and Wadell, 1988; Li et al., 1996]. Therefore, we have demonstrated that these isolates are new genotypes. The letters h, i, j, and k were assigned to each new genotype following our paper [Shiao et al., 1996]. This nomenclature is convenient for following the major changing trend of genotype of Ad3 in Japan. For further differentiation, nomenclature based on restriction endonuclease analysis using 4- or 5-base pair nucleotide-recognizing enzymes or nine enzymes might be taken into consideration [Itakura et al., 1990; Li et al., 1996].

Fifty-eight percent of patients had a diagnosis of EKC and 42% had PCF with various clinical symptoms in this study. There was no evidence that the ocular symptoms associated with adenovirus infection had changed with the appearance of a new Ad3 genotype.

It is important for the prevention of this infectious agent to determine whether the new genotype isolates were introduced from other countries or whether they were derived from former Japanese Ad3 strains. At present, to determine the genetic distance between our Ad strains and other strains, it is necessary to isolate the organism by cell culture, extract viral DNA, and compare genome digestion patterns using many REs. However, isolating viruses is very laborious and comparing the RE digestion profile of one strain with that of the other strains described in the literature is very difficult. Recently, Crawford et al. [1996] demonstrated that the adenovirus hexon sequence contained seven hypervariable regions (HVR) containing serotype specific profiles in each region. Comparison of HVR sequences is expected to reveal the genetic distances between these strains. In the near future, molecular epidemiology based on sequence analysis might be helpful to compare strains isolated in different laboratories.

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